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Flow cytometry data analysis

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An abbreviated version of this protocol was published in Science Immunology in Aug 2020

Natural killer cell immunotypes related to COVID-19 disease severity

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Detailed protocol

MATERIALS AND METHODS

Cell preparation and flow cytometry

1-Samples collection and preparation

i. Venous blood samples were collected in heparin tubes.
ii. PBMCs were isolated using Ficoll gradient in SepMate PBMC isolation tubes (STEMCELL).
iii. PBMCs were washed post-Ficoll in PBS by centrifugation at room temperature (RT)
iv. PBMCs were counted (Countess II, ThermoFisher) and transferred to the 96-well V-bottom plate for the staining.

2-Staining

i. PBMCs were stained fresh with the antibody mix in 96 wells plate V-bottom (for antibodies, see table S3 of the manuscript).
ii. The antibody mixes were composed of previously titrated fluorophore-conjugated antibodies (listed in table S3), FACS buffer (PBS, 2 mM EDTA, 1% FCS) and Brilliant Stain Buffer Plus (1:5, BD Biosciences).
iii. For each sample, master-mixes were prepared: one for extracellular, one for secondary and one for the intracellular staining. Samples were stained in the 50 mL volume for all staining steps.
iv. After each extracellular and secondary staining, cells were washed 3 times in FACS buffer. After the secondary staining, cells were permeabilized for 45 minutes at 4°C in 100 mL of the freshly prepared Foxp3 Fixation/Permeabilization working solution from the eBioscience Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher).
v. Cells were washed 3 times using freshly prepared 1x Permeabilization Buffer from the eBioscience Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher) and stained with the intracellular antibody mix diluted in the 1x Permeabilization Buffer and Brilliant Stain Buffer Plus (1:5, BD Biosciences).
vi. After the staining, cells were fixed with 1% paraformaldehyde for 2 hours at RT, washed, and resuspended in FACS buffer before being acquired on a BD FACSymphony with 355-, 405-, 488-, 561-, and 640-nm lasers.

Notes:
-Live/Dead cell discrimination was performed using fixable Aqua Dead Cell Stain Kit (Invitrogen) added in the first staining step (see table S3).
-To obtain absolute counts of blood NK cells, 50 µl of whole blood from each patient and healthy control were stained with BD Trucount Tubes, according to the instructions from the manufacturer.
-For each batch of experiments (3 in total), a separate vial of PBMCs from the same donor was stained and used as internal control.

3-Manual flow cytometry data analysis

i. FCS3.0 files were exported from the FACSDiva and imported into FlowJo v.10.6.2 for subsequent analysis.
ii. To remove any anomalies, cleaning of the FCS files was done using FlowAI (2.1) with the following parameters: all checks, second fraction FR = 0.1, alpha FR = 0.01, maximum changepoints = 3, changepoint penalty = 500, and dynamic range check side = both.
iii. The compensation matrix for the 28-color flow cytometry panel was generated using single-stained bead controls (UltraComp eBeads, ThermoFisher) and AutoSpill algorithm; such matrix was applied to fcs files.
iv. The resulting dataset was used for the downstream analyses performed both by manual gating and automated analysis (all analyses were performed in FlowJo v.10.6.2).

4-Automated flow cytometry data analysis

i. In the FlowJo workspace, events were first downsampled from the NK gate across all samples using DownSample v.3.2 plugin (fig. S5, A and B).
ii. Clinical parameter categorical values for each sample were added to downsampled populations as metadata to enable identification of these groups.
iii. Samples were concatenated for the downstream analysis.
iv. UMAP 3.1 was run using all parameters from the panel except BV510 (lineage channel: live/dead, CD14, CD15, and CD19) and PE–Cy5 (CD3).

v. PhenoGraph v.2.4 was run using the same parameters from the panel as UMAP (k = 30). Fifteen thousand cells per sample were exported from the NK gate, apart from six patient samples with fewer events where all cells were taken.
vi. When assigning categorical groups formed by different clinical parameters, there was an uneven number of patients represented in each group (e.g., 17 "healthy controls," 12 "viremic," and 12 "nonviremic" patients). As over- and underrepresented input groups were similarly weighted in the PhenoGraph output clusters, the PhenoGraph output clusters were normalized to account for the total number of cells from each input group.

5-Figure generation

Certain figures (i.e. Figs 2-5, S2 and S7) were generated in R (versions 3.6.0 and 3.6.1) with packages such as factoextra (v1.0.5), RColorBrewer (v1.1-2), ggplot2 (v3.2.1 and v3.3.0), tidyr (v.1.0.2), randomcoloR (v.1.1.0.1), reshape2 (v.1.4.3), viridis (v.0.5.1), and pheatmap (v.10.12).

6-Strategy to identify adaptive NK cell expansions

CMV-seropositive healthy controls and COVID-19 patients displaying more than 5% of NKG2C^{+}CD57^{+} cells within their CD56^{dim} NK cell population were considered to have adaptive NK cell expansions (fig. S5A). In all individuals with adaptive expansions, adaptive NK cells displayed higher (>20%) frequencies of either CD57, CD38, or single KIRs compared with the nonadaptive NK cells (and also in one case high NKG2A). One patient displayed a percentage of adaptive NK cells lower than 5% (3.64%) but was included in the expansion group because coexpression of the other phenotypical markers (differential NKG2A, CD57, CD38, and KIR expression) was in line with what has been described for adaptive NK cells.

7-Statistical analysis

Statistical significance was determined using GraphPad Prism v8. For comparisons between three groups, one-way analysis of variance (ANOVA) and Kruskal-Wallis test followed by Dunn's multiple comparisons test were used. For two groups, either parametric or nonparametric matched or nonmatched tests were performed. Where indicated, z score of either median fluorescence intensity (MFI) or percentage of marker expression was calculated as follows: $z = (x-\mu)/\sigma$, where x is the raw score, μ is the mean of sample distribution, and σ is the SD. For categorical comparisons, Fisher's exact test was used. Significant PhenoGraph clusters ($P \leq 0.05$) were determined by chi-square goodness-of-fit tests comparing the relative abundance of each categorical group in each individual PhenoGraph cluster relative to input. For flow cytometry analysis, expression data for multiple markers (both MFI and percentage of protein-expressing cells) derived from gates containing less than 100 events were excluded from analysis. When analyzing the percentage of Ki-67–expressing cells, data derived from gates containing less than 50 cells were excluded from analysis. More details on the exact statistical tests used are mentioned in the respective text/figure legends.

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1. Maucourant, C. , Filipovic, I. , Ponzetta, A. and Björkström, N. (2021). Flow cytometry data analysis. Bio-protocol Preprint. bio-protocol.org/prep828.
2. Maucourant, C., Filipovic, I., Ponzetta, A., Aleman, S., Cornillet, M., Hertwig, L., Strunz, B., Lentini, A., Reinius, B., Brownlie, D., Cuapio, A., Ask, E. H., Hull, R. M., Haroun-Izquierdo, A., Schaffer, M., Klingström, J., Folkesson, E., Buggert, M., Sberg, J. K., Eriksson, L. I., Rooyackers, O., Ljunggren, H., Malmberg, K., Michaëlsson, J., Marquardt, N., Hammer, Q., Strålin, K. and Björkström, N. K.(2020). Natural killer cell immunotypes related to COVID-19 disease severity. Science Immunology 5(50). DOI: 10.1126/sciimmunol.abd6832